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Characterization of scFv-Ig Constructs Generated from the Anti-CD20 mAb 1F5 Using Linker Peptides of Varying Lengths¹

Daming Shan,^{2*} Oliver W. Press,^{*†‡} Theta T. Tsu,^{3§} Martha S. Hayden,^{3§} and Jeffrey A. Ledbetter^{3§}

The heavy (V_H) and light (V_L) chain variable regions of the murine anti-human CD20 mAb 1F5 were cloned, and four single-chain Ab (scFv) molecules were constructed using linker peptides of variable lengths to join the V_H and V_L domains. Three constructs were engineered using linker peptides of 15, 10, and 5 aa residues consisting of (GGGGS)₃, (GGGGS)₂, and (GGGGS)₁ sequences, respectively, whereas the fourth was prepared by joining the V_H and V_L domains directly. Each construct was fused to a derivative of human IgG1 (hinge plus CH2 plus CH3) to facilitate purification using staphylococcal protein A. The aggregation and CD20 binding properties of these four 1F5 scFv-Ig derivatives produced were investigated. Both size-exclusion HPLC column analysis and Western blots of proteins subjected to nonreducing SDS-PAGE suggested that all four 1F5 scFv-Ig were monomeric with m.w. of ~55 kDa. The CD20 binding properties of the four 1F5 scFv-Ig were studied by ELISA and flow cytometry. The 1F5 scFv-Ig with the 5-aa linker (GS1) demonstrated significantly superior binding to CD20-expressing target cells, compared with the other scFv-Ig constructs. Scatchard analysis of the radiolabeled monovalent GS1 scFv-Ig revealed a binding avidity of $1.35 \times 10^8 \text{ M}^{-1}$ compared with an avidity of $7.56 \times 10^8 \text{ M}^{-1}$ for the native bivalent 1F5 Ab. These findings suggest that the GS1 scFv-Ig with a short linker peptide of ~5 aa is the best of the engineered constructs for future studies. *The Journal of Immunology*, 1999, 162: 6589–6595.

Monoclonal Abs and radioimmunoconjugates targeting the CD20 Ag have recently been shown to induce remissions in 50–95% of patients with relapsed B cell non-Hodgkin's lymphomas (1–4). Despite the clinical promise of anti-CD20-targeted therapy, it must be acknowledged that currently available Ab constructs are imperfect, and that most patients who initially respond to anti-CD20 Ab immunotherapy will eventually relapse with lymphoma. Many investigators have hypothesized that anti-CD20 Abs might be significantly improved by genetic engineering approaches aimed at facilitating penetration into tumor sites. Our group has recently begun a series of investigations aimed at developing superior anti-CD20 Abs by cloning and modifying the Ag-combining portion of the 1F5 mAb. The smallest Ab fragment that still contains the entire Ag binding site is the Fv

fragment, consisting of the variable domains of the heavy (V_H)⁴ and light (V_L) chains (Fig. 1). The variable domains of Fv fragments are not associated by covalent bonds, and consequently the domains tend to dissociate at low protein concentrations (5). One of the approaches to improve the stability of the Fv domain association involves construction of single-chain Abs (scFvs) in which the two variable domains are linked via a short flexible peptide (Fig. 1). Previous studies indicate that the lengths and sequences of the linker peptide can significantly affect the properties of scFvs (6–15). Peptide sequences from known protein structures have been used as linkers, the lengths and conformations of which are compatible with bridging the variable domains of scFvs without serious steric interference. The most widely used linker designs have sequences consisting primarily of stretches of glycine (G) and serine (S) residues. These small, polar amino acids are optimal for the linker peptides because hydrophilic amino acids allow hydrogen bonding to the solvent, and glycines provide the necessary flexibility (6). These properties prevent the penetration of the linker peptide into the hydrophobic interface formed in the association of the domains. An example of this type of linker is the (GGGGS)₃ peptide designed by Huston et al. (9).

Several studies have focused on the effect of different lengths of linker peptides on the properties of scFvs (7, 8, 10, 12–15). These studies suggest that direct linking of the domains or use of linker peptides which are too short to allow pairing of the domains on the same chain favor pairing between domains in two adjacent chains. In this way, small, dimeric, fully functional Ab fragments (“diabodies”) have been produced. To assess the relative merits of scFvs compared with intact anti-CD20 Abs, we have constructed four scFv-Ig fusion proteins of the anti-CD20 mAb 1F5 using amino acid linkers of 0, 5, 10, and 15 residues, respectively. The properties of these scFv-Ig were then investigated.

Departments of *Biological Structure and †Medicine, University of Washington, Seattle, WA 98195; ‡Fred Hutchinson Cancer Research Center, Seattle, WA 98104; and §Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA 98121

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² Address correspondence and reprint requests to Dr. Daming Shan, University of Washington Medical Center, Division of Medical Oncology, Box 356043, Seattle, WA 98195-6043. E-mail address: dmsan@u.washington.edu

³ Current address: Xcyte Therapies, 2203 Airport Way South, Suite 300, Seattle, WA 98134.

⁴ Abbreviations used in this paper: V_H , variable domain of heavy chain; V_L , variable domains of light chain; scFvs, single-chain Abs; scFv-Ig, single-chain Abs and Ig fusion protein; G, glycine; S, serine; GS0, V_H domain with 0-aa linker peptides; GS1, V_H domain with 5-aa linker peptides; GS2, V_H domain with 10-aa linker peptides; and GS3, V_H domain with 15-aa linker peptides; GAM, goat anti-mouse IgG Ab; GAH, goat anti-human IgG Ab; PI, propidium iodide.

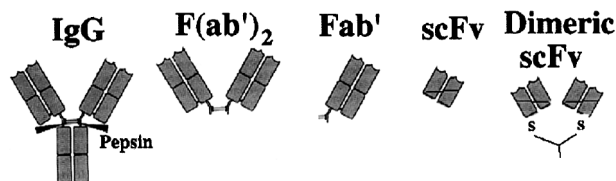


FIGURE 1. Schematic representation of the structures of intact IgG mAb, F(ab')₂, Fab', and scFv.

Materials and Methods

Amplification of V_H and V_L by PCR

PCR reactions were conducted in 100 μ l volumes containing 20 μ mol of each dNTP, 50–75 pmol primers, 1 ng template, and *Taq* polymerase (Stratagene, Torrey Pines, CA) in the polymerase buffer supplied by the manufacturer. PCR was conducted with 33 cycles of 30 s denaturation at 94°C, 1.5 min annealing at 45°C, and 1.5 min extension at 72°C. Amplified DNA was digested with restriction endonucleases *Bcl*I and *Sac*I for V_H and *Sac*I and *Sac*I for V_L (Boehringer Mannheim, Indianapolis, IN). Digested and purified DNA was cloned into the PUC19 plasmid using those sites. DH5a *Escherichia coli* cells were transformed with the plasmid and DNA. Miniprep DNA was purified using GeneClean (Bio101, La Jolla, CA). DNA from three of four clones of each V_H and V_L were sequenced to determine the consensus sequence by automated sequencing using ³²P-labeled dNTP.

Three-way ligation

Aliquots (5 μ l) of miniprep DNA from PUC19 plasmids carrying V_L and V_H gene fragments were digested with a mixture of *Sac*I and *Sac*I restriction enzymes for V_L and *Bcl*I and *Sac*I for V_H. The digests were incubated for 2 h at 37°C (56°C for *Bcl*I), and DNA fragments were separated by electrophoresis on minigels of 0.75% Seakem agarose (FMC Bioproducts, Rockland, ME). DNA bands corresponding to V_L and V_H fragments were cut from gels and purified using GeneClean. DNA fragments encoding V_H and V_L were cloned into modified PUC19 at the same time. The PUC19 plasmid was modified as follows: the *Hind*III site at the 5' end of the stuffer region was used to insert a *Hind*III-*Sac*I fragment containing the anti-L6 Ig light chain leader sequence for secretion of scFv-Ig, and the *Xba*I site at the 3' end of the stuffer region was used to insert the DNA sequence encoding the hinge-CH2-CH3 portion of human IgG1 in which hinge cysteines had been mutated to serines to favor the production of monomeric scFv-Ig. The cloned DNA fragment was then cut by *Xba*I and *Hind*III, purified by GeneClean, and inserted into the pCDM8 vector for expression.

Transient transfection in COS cells

COS cells were seeded at a density of 10⁶ cells per 10-cm diameter culture dish 18–24 h before transfection. Plasmid DNA was added (~15 μ g/dish) in a volume of 5 ml of serum-free DMEM containing 0.1 mM chloroquine and 600 μ g/ml DEAE dextran, and cells were incubated for 3–3.5 h at 37°C. Transfected cells were then briefly treated (~2 ml) with 10% DMSO in PBS and incubated at 37°C for 16–24 h in DMEM containing 10% FCS. The culture medium was removed ~24 h after transfection and replaced with serum-free DMEM (10 ml/dish). Incubation was continued for 3 days at 37°C, and then the spent medium was collected and fresh serum-free medium was added. After an additional 3 days at 37°C, the spent medium was again collected and cells were discarded. Culture supernatants were tested for the presence of scFv-Ig proteins and for specific binding activity of those proteins as described below.

Purification of 1F5 scFv-Ig

MC106/p3 clones, which contained host pCDM8 vectors and produced functional scFv-Ig, were initially identified by screening supernatants of small-scale preparations for binding to Ramos cells using FACS. Then, large-scale transfections were set up to generate sufficient quantities of fusion proteins for purification and experimental testing. Subsequently, 800 ml of serum-free supernatants were collected over a 6-day culture period. Cellular debris was removed by low-speed centrifugation, and clarified supernatants were applied to columns of immobilized protein A at a rate of 1 ml/min. The columns were rinsed with PBS and proteins were eluted with 0.1 M glycine/0.15 M NaCl, pH 2.7, neutralized immediately with 1.0 M Tris-HCl, pH 9.0, and then dialyzed against PBS at 4°C.

Detection of 1F5 scFv-Ig by Western blotting

1F5 scFv-Ig fusion proteins were immunoprecipitated from culture supernatants, and samples were electrophoresed on linear gradient (6–15%) acrylamide gels at 200 V for 4–5 h. Gels were blotted to nitrocellulose membranes using a Western semidry transfer apparatus (Ellard Instrumentation, Seattle, WA) at 20 V (3 mA/cm²) for 1 h. Blots were blocked with blocking buffer (2% nonfat milk plus 0.1% Tween in PBS) and then incubated with goat anti-human IgG (GAH) conjugated to alkaline phosphatase (Boehringer Mannheim) in blocking buffer. Blots were washed three times in blocking buffer and developed in Western Blue (Promega, Madison, WI).

Gel filtration analysis of 1F5 scFv-Ig

The m.w. of 1F5 scFv-Ig before and after purification with protein A were analyzed by gel filtration chromatography on a Waters 7.8 $\times$ 300 mm 300SW HPLC column (Waters, Milford, MA). Samples were chromatographed at a flow rate of 0.35 ml/min in 10 mM Tris-HCl, pH 7.5, 10 mM potassium phosphate, and 0.15 M NaCl. The protein standards used to determine the relative molecular masses of 1F5 scFv-Ig were as follows: blue dextran, 2 $\times$ 10⁶ Da; ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158 kDa; BSA, 67 kDa; OVA, 43 kDa; chymotrypsinogen, 25 kDa; and RNase, 13.7 kDa.

Binding activity of 1F5 scFv-Ig detected by ELISA

A total of 10⁵ Ramos cells/well were added to U-shaped 96-well microtiter plates (Falcon 3072, Becton Dickinson, Lincoln Park, NJ). Plates were blocked with PBS containing 5% BSA, and purified 1F5 scFv-Ig fusion protein or 1F5 was added. Following a 1-h incubation at 4°C and removal of unbound material, bound protein was detected with GAH or goat anti-mouse IgG Ab (GAM) labeled with HRP (American Qualex, La Miranda, CA) in blocking buffer for 45 min at 4°C. Unbound conjugates were removed by washing with PBS plus Tween 20, and o-phenylenediamine (Sigma, St. Louis, MO) in citrate buffer was added to the wells. The reaction was stopped with 2 M HCl, and the OD was measured at 490 nm with a Titertek multiwell plate reader (Titertek instruments, Huntsville, AL).

Binding activity of 1F5 scFv-Ig detected by flow cytometry

Ramos cells were incubated with COS supernatants/PBS, purified 1F5 scFv-Ig fusion proteins, or purified intact 1F5 Ab in staining medium (RPMI 1640 plus 5% FBS plus 0.1% sodium azide) for 45 min on ice. Cells were washed, and bound scFv-Ig fusion proteins were detected with GAH conjugated to FITC (Tago, Burlingame, CA) for 30 min on ice. Bound murine 1F5 Ab was detected using a FITC-labeled GAM (Tago). The cells were washed with ice-cold staining medium and fluorescence was analyzed using a flow cytometer (Becton Dickinson, Mountain View, CA).

Radioiodination of Abs and Scatchard analysis of binding

1F5 and 1F5 scFv-Ig were labeled with Na¹²⁵I by the Iodogen method. Ab (100 μ g/ml) was incubated with 0.5 mCi of Na¹²⁵I in glass tubes coated with 10 μ g of Iodogen (Pierce, Rockford, IL) for 5 min at room temperature. Free Na¹²⁵I was removed by chromatography on a Pharmacia PD-10 column (Pharmacia, Piscataway, NJ). Eluted fractions containing ¹²⁵I-labeled Ab were pooled and stored at 4°C.

Cell binding assays were performed in 1.5 ml microcentrifuge tubes. Labeled Abs were serially diluted in tissue culture medium (RPMI 1640 plus 2% BSA plus 0.02% sodium azide) and 100 μ l of each concentration mixed with an equal volume of serial cell dilutions in microcentrifuge tubes. A 100- μ l aliquot of each Ab dilution was also counted in a Beckman γ 5500 counter (Beckman Instruments, Palo Alto, CA) to determine the sp. act. of the labeled Abs and the total amount of Ab added for each dilution. The immunoreactive fraction of each Ab preparation was determined by Lineweaver-Burk analysis using a fixed concentration of Ab (40 ng/ml) incubated with varying numbers of target cells (10⁷–10⁵/well) as described by Lindmo (16). Scatchard analysis was performed by incubating serial dilutions of Ab (10 μ g/ml to 10 ng/ml) with a fixed number of cells (2 $\times$ 10⁵). Tubes were incubated at 37°C for 1 h. The target cells were then washed three times by centrifugation, and bound activity was counted. Scatchard plots were then used to determine binding avidities as described by Trucco (17).

Flow cytometric analysis of apoptosis using propidium iodide (PI) staining

Flow cytometric analysis of cellular DNA was performed following PI staining according to the method of Fried et al. (18). Briefly, 10⁶ Ramos

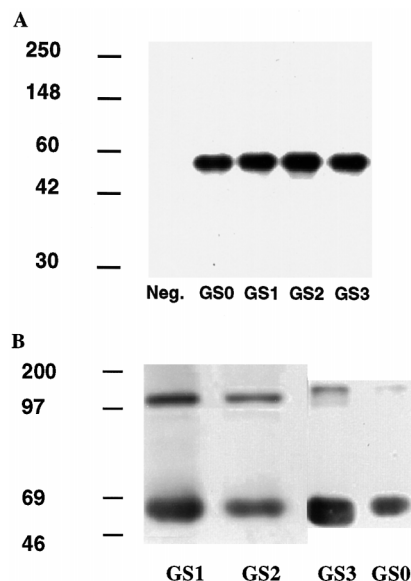


FIGURE 4. Detection of 1F5 scFv-Ig by Western blot analysis. 1F5 scFv-Ig from COS cell supernatants were detected by probing Western blots using alkaline phosphatase-conjugated GAH after separating these proteins on reducing (A) and nonreducing (B) SDS-PAGE gels. Neg., Supernatants from cells transfected with pCDM8 vector without inserts.

shown). These results demonstrated that no significant aggregation occurred with any of the 1F5 scFv-Ig when analyzed by HPLC. The presence of a minor degree of dimer formation on nonreducing SDS-PAGE gels was presumably due to association of the CH3 domains of the Ig tail, as has been reported previously (20).

Detection of binding activity of 1F5 scFv-Ig by flow cytometry and ELISA

The relative CD20 binding activities of these new 1F5 scFv-Ig constructs were compared in flow cytometric and ELISA assays using CD20-expressing Ramos cells and control Jurkat cells. In the former assay, the binding activity was tested by adding 1F5 scFv-Ig constructs to Ramos cells followed by staining with FITC-conjugated GAH as a second step reagent and analyzing by flow cytometry. The results indicated that 1F5 scFv-Ig bind specifically to CD20 expressed on Ramos cells because nonspecific binding of 1F5 scFv-Ig was not found with Jurkat cells, which do not express the target CD20 Ag (Fig. 6). The order of binding activity was $(GGGGS)_1 > (GGGGS)_2 > (GGGGS)_0 > (GGGGS)_3$ (Fig. 6). The specificity of binding was also confirmed using a competitive

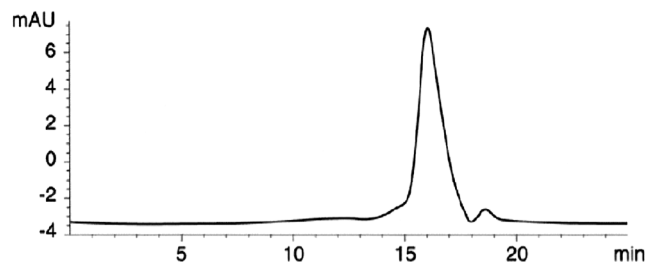


FIGURE 5. The size of 1F5 scFv-Ig constructs as determined by HPLC analysis. COS cell supernatants containing 1F5 scFv-Ig or purified 1F5 scFv-Ig were eluted through a size-exclusion HPLC column. Results are presented for the 1F5 scFv-Ig constructed with a 5-aa residue linker after affinity purification using protein A. Similar results were observed for all other 1F5 scFv-Ig before and after purification. mAU, Milli absorbency unit.

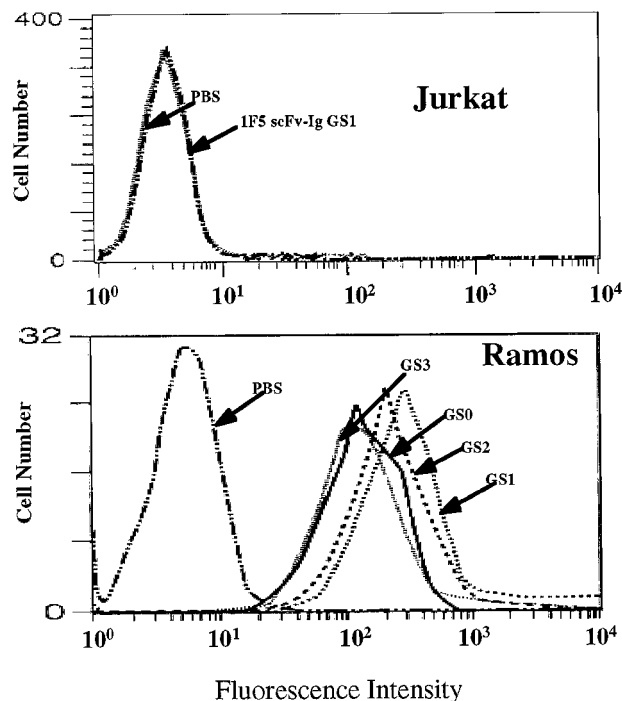


FIGURE 6. 1F5 scFv-Ig binding demonstrated by flow cytometry. A total of 10^5 Jurkat or Ramos cells were incubated with PBS or 50 $\mu\text{g/ml}$ 1F5 scFv-Ig for 45 min at 4°C followed by washing and incubation with FITC-labeled GAH for 30 min at 4°C. Cells were analyzed by flow cytometry. Data are representative of three concordant experiments.

binding assay employing unlabeled 1F5 scFv-Ig to block binding of fluorescein-labeled intact 1F5 (data not shown). The mean fluorescence intensity of Ramos cells incubated with FITC-1F5 declined from channel 31.02 in the absence of blocking 1F5 scFv-Ig to channel 18.64 in the presence of unlabeled 1F5 scFv-Ig. The relative binding activities of the different 1F5 scFv-Ig were confirmed in an ELISA assay, performed by adding 25 μl of various concentrations (12.5–100 $\mu\text{g/ml}$) of 1F5 scFv-Ig to 10^5 Ramos cells, followed by the addition of a peroxidase-conjugated GAH second Ab (Fig. 7). Thus, all of the scFv-Ig constructs retained

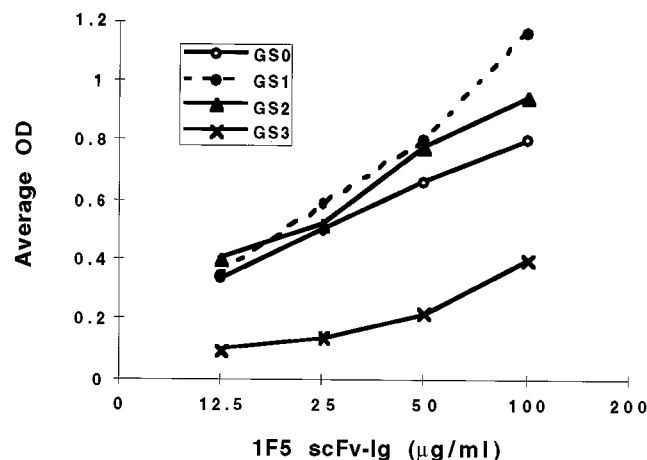


FIGURE 7. Binding activity of 1F5 scFv-Ig demonstrated by ELISA. A total of 10^5 Ramos cells were incubated with different concentrations of 1F5 scFv-Ig, followed by incubation with a peroxidase-conjugated GAH second Ab. The results are presented as the average OD of duplicate wells, after subtracting the OD of control wells incubated only with the secondary Ab. Data are representative of three concordant experiments.

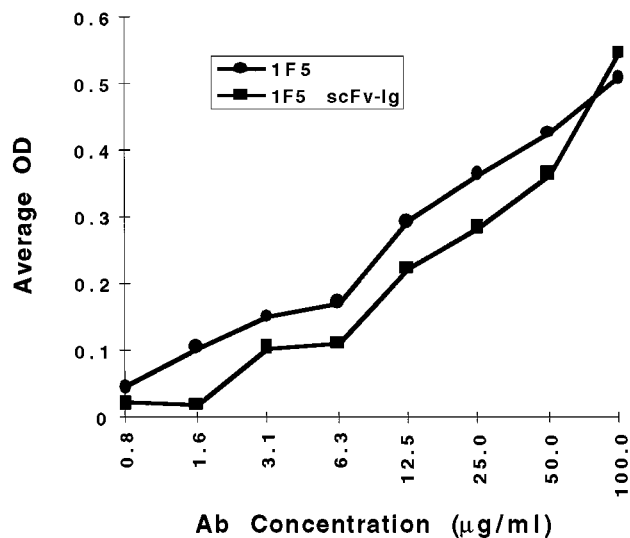


FIGURE 8. Binding activity of 1F5 scFv-Ig compared with 1F5. A total of 10^5 Ramos cells were incubated with various concentrations of 1F5 scFv-Ig (GS1) or 1F5, followed by incubation with a peroxidase-conjugated GAH or GAM secondary Ab. The results are presented as the average OD of duplicate wells, after subtracting the OD of control wells incubated only with the secondary Ab. Data are representative of three concordant experiments.

CD20 binding activity, but the binding activities were strongly influenced by the length of the linker.

Comparison of binding activity of 1F5 scFv-Ig with intact 1F5

The binding activity of the best 1F5 scFv-Ig construct (GS1) was further compared with the binding of the intact anti-CD20 mAb 1F5 by ELISA, flow cytometry, and radiolabeled cell binding assays. As shown in Fig. 8, the binding activity of the monomeric 1F5 scFv-Ig was comparable, though slightly inferior to that of the intact bivalent 1F5. Similar results were obtained using flow cytometry (data not shown). A more quantitative analysis of comparative binding was obtained by radiolabeling both the GS1 1F5 scFv-Ig construct and the intact 1F5 Ab and performing a standard Scatchard binding analysis. Because soluble CD20 was not available, the binding of ^{125}I -labeled 1F5 scFv-Ig and intact 1F5 were measured using CD20-bearing Ramos cells, and data were analyzed by Scatchard analysis by the method of Trucco (17). Using this approach, the binding avidity of the 1F5 scFv-Ig was estimated to be $1.35 \times 10^8 \text{ M}^{-1}$, whereas the binding avidity of the bivalent 1F5 Ab was $7.56 \times 10^8 \text{ M}^{-1}$.

Induction of apoptosis in human malignant B cell lines by 1F5 scFv-Ig

The functional activities of 1F5 scFv-Ig were subsequently assessed by measuring the capability of these constructs to induce apoptosis of Ramos cells after cross-linking CD20 with 1F5 scFv-Ig plus GAH. Our group (21) and others (22) have previously demonstrated that intact anti-CD20 mAbs reproducibly induce apoptosis of malignant human B cells if the CD20 molecule on mAb-coated cells is cross-linked using GAM or FcR-bearing accessory cells. To investigate whether 1F5 scFv can induce apoptosis in a similar fashion, we treated Ramos cells with 1F5 scFv-Ig in the presence and absence of GAH and then stained cells with PI followed by flow cytometry analysis. As depicted in Fig. 9, 1F5 scFv-Ig could induce a small amount of apoptosis in Ramos cells when used alone ($p < 0.05$), but was more effective at inducing

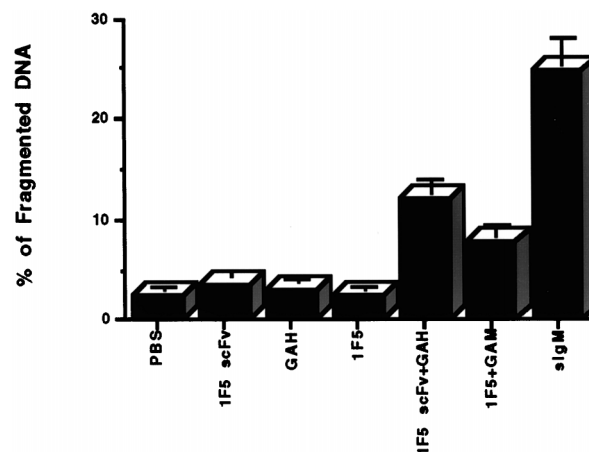


FIGURE 9. Apoptotic effect of 1F5 scFv-Ig on Ramos cells as demonstrated by PI staining. A total of 10^6 cells/ml were incubated with 5 $\mu\text{g/ml}$ of 1F5 scFv-Ig GS1 in the presence or the absence of 50 $\mu\text{g/ml}$ GAH or with 10 $\mu\text{g/ml}$ 1F5 in the presence or the absence of 50 $\mu\text{g/ml}$ GAM for 24 h. Cell nuclei were stained with PI and analyzed by flow cytometry. Hypodiploid DNA peaks corresponding to apoptotic nuclei were quantified. Cells incubated with PBS or 50 $\mu\text{g/ml}$ GAH were used as negative control, and cells incubated with 10 $\mu\text{g/ml}$ anti-sIgM Ab were used as positive control. Data are representative of three concordant experiments.

apoptosis when further cross-linked by a secondary GAH Ab ($p < 0.01$), similar to our observations with intact murine 1F5.

Discussion

Both unmodified and radiolabeled anti-CD20 mAbs have been successfully employed in the immunotherapy of B cell lymphomas (1–3, 23–25). Tumor remissions have been observed in up to 50% of lymphoma patients treated with unmodified mAbs (1, 26) and in 72–95% of patients receiving radiolabeled mAbs (2–4). However, slow and heterogeneous penetration of these intact mAbs into tumor sites remains a limiting factor for optimal immunotherapy. scFvs are smaller than intact mAbs and have been shown to penetrate tumors faster, more deeply, and more homogeneously in vivo (27–30). In addition, their small size renders them less immunogenic than intact mAbs. Despite these advantages, it must be acknowledged that scFvs often achieve lower intratumoral concentrations and exhibit shorter tumor retention than intact mAbs because of their lower binding affinities (27, 31, 32). One of the approaches to improve the scFv affinity has been to employ optimal linker peptides, which increase affinity by affecting the structure and assembly of scFvs (6, 8, 9). In this study, we have constructed four 1F5 scFv-Ig with different lengths of linker peptides to explore whether the binding of 1F5 scFv-Ig will be affected as reported in other Ab systems (33). Our results indicate that 1F5 scFv-Ig with shorter linker peptides (5 aa) have higher binding activities than 1F5 scFv-Ig with longer linker peptides. However, 1F5 scFv-Ig with V_L and V_H domains connected together directly without a linker peptide do not show a further improvement of binding activity. Our results also demonstrate that the binding activity of the monovalent 1F5 scFv-Ig is comparable, though slightly inferior, to that of the parental intact bivalent 1F5 Ab. This modest decrement in binding avidity of the scFv-Ig compared with the intact Ab is expected based on the difference in valency of binding, and is consistent with observations by many other workers comparing other types of scFv with their parental Abs (11, 33).

1F5 is one of a panel of mAbs that have been produced recognizing the CD20 pan B cell Ag (34), and it was the first anti-CD20

mAb tested in lymphoma patients (26). Anti-CD20 mAbs are attractive for immunotherapy of B cell lymphomas because 1) anti-CD20 Abs do not internalize after cell surface binding, 2) anti-CD20 Abs are not shed from the cell surface, 3) CD20 is expressed at a high density on >95% of all B cell lymphomas, and 4) the antigenic density of CD20 is relatively homogenous from one tumor cell to another (35). To further improve the promising therapeutic effects of anti-CD20 mAbs by enhancing penetration into tumor sites, smaller fragments of these mAbs have been suggested to replace intact mAbs (27, 36, 37). However, preliminary efforts to generate 1F5 F(ab')₂ enzymatically have been laborious and yields have been disappointing (our unpublished observations). Fab of these mAbs have been produced but their monovalent binding and lower avidities have impeded clinical applications (27, 36, 37). These disadvantages have induced us to pursue genetic approaches to synthesize new Ab derivatives. Ultimately, these efforts are focused on making these special Abs smaller, while retaining bivalent binding properties to make their affinities comparable to intact mAbs.

Recombinant fragments with two binding sites have been made in several ways, for example, by chemical cross-linking of the hinge cysteine residues (38) or by including a C-terminal peptide that promotes dimerization (39, 40). More recently, single-chain Abs have been reported by many research groups to have the tendency to form dimers and higher molecular multimers when the linker peptides are short, especially shorter than 15 aa residues (7, 15, 33). The explanation for this phenomenon is that short linkers presumably constrain the folding and appropriate association of V_H and V_L located on the same polypeptide, which expose hydrophobic residues normally concealed by the V_H-V_L interface, therefore increasing the likelihood of intermolecular associations and resulting in multimerization.

In our study, both Western blotting and HPLC data indicated that most of the functionally active 1F5 scFv-Ig were monomeric despite the differences in their linker peptide lengths. This was not expected because most of the other scFv produced with short linker peptides tend to form dimers or multimers (11, 33). A possible explanation for our findings is that the Ig tail may limit the aggregation of 1F5 scFv-Ig. Although a small amount of dimerization was found on nonreducing SDS-PAGE gels, this was probably due to intermolecular associations via the Ig CH3 domain as has been reported previously for scFv-Ig constructs (20).

One potential explanation for the apparent increase in the CD20 binding activity of the 1F5 scFv-Ig constructs synthesized with shorter linker peptides may be that they formed dimers or multimers in culture, leading to an increase in their binding valency. However, this hypothesis is apparently not consistent with our Western blot and HPLC experiments demonstrating <30% dimerization. A possible explanation for this discrepancy is that dimerization may have occurred after binding of monomeric scFv-Ig to the CD20 Ag on the cell surface due to local concentration effects promoting the formation of dimers and higher m.w. forms as has been reported for other scFvs (7, 15). This type of multimerization has been reported to be most likely for scFv recognizing Ags present at high density on a solid phase such as cells (7, 15). In support of this hypothesis, our experiments show that the slope of 1F5 scFv-Ig GS1 binding in our ELISA assay exhibits a cooperative binding effect in contrast to the slope of the 1F5 scFv-Ig GS3 (Fig. 7). An alternative explanation for the superior binding of the short linker construct GS1 may be that the 5-aa linker caused subtle conformational changes leading to a slightly altered binding pocket, causing a better "fit" with the CD20 Ag.

A major reservation to the substitution of scFv constructs for intact mAbs in immunotherapeutic approaches involves the possi-

ble loss of anti-tumor effect or functions including complement fixation, Ab-dependent cellular cytotoxicity, and induction of apoptosis. Although the first two of these functions are dependent on the physical presence of the Fc portion of the Ig molecule, apoptosis induction is triggered by binding to the target Ag and might be preserved with scFv constructs. Our results indicate that this may be the case with the 1F5 scFv described in this manuscript, though optimal induction of apoptosis required amplification by cross-linking the scFv with a GAH secondary reagent. Because the human IgG1 tail used in our scFv-Ig constructs can bind Fc receptors, this apoptotic effect may be augmented in vivo by cross-linking mediated by FcR-bearing accessory cells. We conclude that the 5-aa GS1 1F5 scFv construct merits further investigation for targeted therapy of CD20-expressing B cell malignancies including applications involving radionuclide conjugates and pretargeting strategies using scFv-streptavidin conjugates in combination with biotin-⁹⁰Y secondary reagents. In addition, further structural modifications are underway to generate dimeric scFv, which may prove superior to the present constructs.

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